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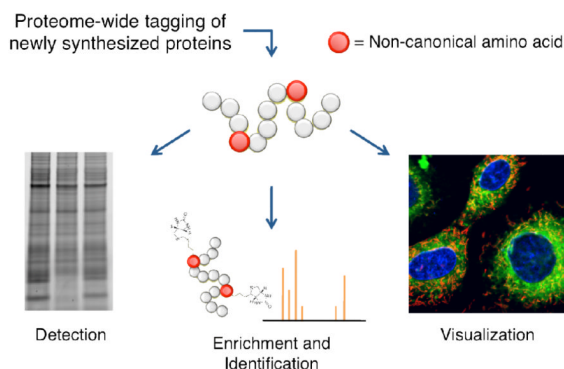
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Non-Canonical Amino Acids in the Interrogation of Cellular Protein Synthesis

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Abstract



CONSPECTUS—Proteins in living cells can be made receptive to bioorthogonal chemistries through metabolic labeling with appropriately designed, non-canonical amino acids (ncAAs). In the simplest approach to metabolic labeling, an amino acid analog replaces one of the natural amino acids specified by the protein’s gene (or genes) of interest. Through manipulation of experimental conditions, the extent of the replacement can be adjusted. This approach, often termed residue-specific incorporation, allows the ncAA to be incorporated in controlled proportions into positions normally occupied by the natural amino acid residue. For a protein to be labeled in this way with an ncAA, it must fulfill just two requirements: (i) the corresponding natural amino acid must be encoded within the sequence of the protein at the genetic level, and (ii) the protein must be expressed while the ncAA is in the cell.

Because this approach permits labeling of proteins throughout the cell, it has enabled us to develop strategies to track cellular protein synthesis by tagging proteins with reactive ncAAs. In procedures similar to isotopic labeling, translationally active ncAAs are incorporated into proteins during a “pulse” in which newly synthesized proteins are tagged. The set of tagged proteins can be distinguished from those made before the pulse by bioorthogonally ligating the ncAA side chain to probes that permit detection, isolation, and visualization of the labeled proteins.

Non-canonical amino acids with side chains containing azide, alkyne, or alkene groups have been especially useful in experiments of this kind. They have been incorporated into proteins in the form of methionine analogs that are substrates for the natural translational machinery. The selectivity of the method can be enhanced through the use of mutant aminoacyl transfer RNA synthetases (aaRSs) that permit incorporation of ncAAs not used by the endogenous biomachinery. Through expression of mutant aaRSs, proteins can be tagged with other useful

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ncAAs, including analogs that contain ketones or aryl halides. High-throughput screening strategies can identify aaRS variants that activate a wide range of ncAAs.

Controlled expression of mutant synthetases has been combined with ncAA tagging to permit cell-selective metabolic labeling of proteins. Expression of a mutant synthetase in a portion of cells within a complex cellular mixture restricts labeling to that subset of cells. Proteins synthesized in cells not expressing the synthetase are neither labeled nor detected. In multicellular environments, this approach permits the identification of the cellular origins of labeled proteins.

In this Account, we summarize the tools and strategies that have been developed for interrogating cellular protein synthesis through residue-specific tagging with ncAAs. We describe the chemical and genetic components of ncAA-tagging strategies and discuss how these methods are being used in chemical biology.

INTRODUCTION

Shortly after the discovery of deuterium by Urey and coworkers, Rudolph Schoenheimer suggested that isotopically tagged cellular constituents could be used to trace and identify the products of metabolic reactions.¹ In 1938, Schoenheimer reported the first metabolic labeling of proteins with an isotopically tagged amino acid: in rats fed ¹⁵N-labeled tyrosine, it was found that a fraction of the amino acid was retained within the animal in the form of protein.² In the following decades, delineation of the mechanism of protein synthesis would stimulate the prospect that other non-naturally occurring amino acids might be incorporated into proteins. By 1956, this idea was validated by the demonstration that selenomethionine (Se-Met, Fig. 1) could be used by bacterial cells to make proteins.³ Today, hundreds of translationally active ncAAs have been identified, and recent progress in the incorporation of reactive ncAAs, combined with developments in bio-orthogonal chemistry, have led to new ways to trace the lives of proteins.

INCORPORATION OF ncAAs INTO PROTEINS

Codons are assigned to amino acids through selective aminoacylation of transfer RNAs (tRNAs) followed by accurate base-pairing between charged tRNAs and messenger RNAs. Amino acids are assigned to individual tRNAs by the aminoacyl-tRNA synthetases (aaRSs). Manipulation of the aminoacylation step to direct the addition of ncAAs to tRNA has facilitated the incorporation of ncAAs into proteins in both site-specific and residue-specific fashion.

Site-Specific Incorporation

Site-specific incorporation methods allow the investigator to insert a single ncAA at a predetermined position in a recombinant protein. In the most common approach, a TAG stop codon is introduced into the gene of interest. Translation of the full-length protein is enabled by introduction of a suppressor tRNA charged with the ncAA. Introduction of the aminoacyl-tRNA is accomplished either by injection of a chemically misacylated tRNA⁴ or by expression of an orthogonal aaRS/tRNA pair.⁵

In site-specific insertion, the ncAA is incorporated into a selected protein at a predetermined site. Since measures can be taken to ensure that incorporation at the selected site does not interfere with protein structure, site-specific approaches are ideal for introducing ncAAs into proteins with minimal perturbation. However, it should be noted that non-target proteins are labeled by incidental “read-through” on other transcripts that include the blank codon.

Site-specific incorporation of ncAAs is a powerful technology with important applications in protein engineering, biochemistry and biophysics.⁶ Site-specific incorporation of reactive side chains has been used to create useful protein conjugates,⁷ and the incorporation of biophysical probes has revealed atomistic details of protein structure and function in bacteria, yeast,⁸ and mammalian cells.⁹ For a comprehensive review of site-specific technology, see Liu and Schultz.⁶

Residue-Specific Incorporation

Under normal physiological conditions, the aaRSs catalyze high fidelity aminoacylation of cognate tRNAs with a collective error rate of $\sim 10^{-4}$.¹⁰ However, the specificity of the synthetases can be overcome by reducing the concentrations of their natural amino acid substrates and supplementing the cellular medium with substrate analogs. This approach has been used to achieve residue-specific incorporation of a wide variety of useful ncAAs.

In residue-specific incorporation, an ncAA serves as a surrogate for a natural amino acid and is inserted into cellular proteins at multiple sites. The rate at which the ncAA replaces the natural residue can be varied to suit the application of interest. Under certain conditions, quantitative substitution can be achieved; if partial replacement is preferred (e.g., for protein tagging with minimal perturbation of structure and function), the extent of incorporation can be set by controlling the concentrations of the canonical and non-canonical amino acids in the medium.¹¹

For a protein to be labeled in residue-specific fashion, it must fulfill just two requirements: (1) the corresponding natural amino must be encoded within sequence of the protein at the genetic level, and (2) the protein must be expressed while the ncAA is in the cell. Depending on the properties of the ncAA, the entire proteome may be subject to tagging. Residue-specific methods are therefore ideally suited for interrogating cellular protein synthesis.

Throughout the last decade, ncAAs with reactive side chains have been of particular interest to our laboratory. In combination with rapid progress in the development of bio-orthogonal reactions,¹² the chemistry of ncAAs is providing powerful new tools for tagging and modifying proteins. Although reactive ncAAs have many potential uses in biology and medicine, this Account will focus on their application in proteomic analysis of cells, tissues, and organisms.

Azides and Alkynes in Metabolic Profiling

The discovery and development of new bio-orthogonal reactions has transformed chemical biology. The use of functionalized analogs of biomolecular building blocks, or “chemical reporters,” has permitted the tagging and tracking of cellular components including glycans¹³, lipids¹⁴, proteins¹⁵, RNA¹⁶, and DNA¹⁷. Azides and alkynes have assumed leading roles in many chemical reporter strategies; these functional groups are small, stable under biologically relevant conditions and virtually absent from cells and tissues. Furthermore, many azide and alkyne analogs of cellular building blocks can be incorporated into target biomolecules by the normal biosynthetic machinery of the cell. These analogs mimic the fates of their natural counterparts and can be used to track cellular metabolism. For detection, both azides and alkynes can be revealed by Cu(I) catalyzed azide-alkyne cycloaddition^{18, 19} (CuAAC) to appropriately functionalized probes (haptens, fluorescent dyes, or affinity reagents). The azide is especially versatile; in addition to CuAAC, azides are also susceptible to the Staudinger ligation²⁰ and to strain-promoted ligation to cyclooctynes and their derivatives.^{21, 22, 23}

We have developed a set of azide- and alkyne-functionalized ncAAs to tag proteins and track cellular protein synthesis. In 2000, we described incorporation of the Met analog

homopropargylglycine (Hpg) into proteins expressed in *Escherichia coli*.²⁴ Although kinetic assays with purified *E. coli* methionyl-tRNA synthetase (MetRS) showed Hpg to be activated roughly two orders of magnitude more slowly than Met, quantitative replacement of Met by Hpg was observed when the marker protein DHFR was expressed in a Met-auxotrophic host strain. Shortly thereafter, in collaboration with the Bertozzi laboratory, we reported incorporation of azidohomoalanine (Aha), second Met surrogate.²⁵ Like Hpg, Aha is activated more slowly than Met, but can replace Met essentially quantitatively in recombinant proteins. In our first report on Aha, we demonstrated that azide-labeled proteins could be selectively modified by the Staudinger ligation.

PROFILING PROTEIN SYNTHESIS

The cellular proteome dictates cell identity, state, and function; the proteins expressed in the cell drive processes such as cell division and differentiation, and changes in protein expression allow cells and organisms to adapt to signals and stresses. In order to understand how cells execute these and other functions, the proteins involved in each process – including those that are newly synthesized, modified, or degraded – must be identified. Because the complexity of the proteome frustrates such studies, methods that examine subsets of the proteome (e.g., “phosphoproteomics,”²⁶ “glycoproteomics,”¹³ and “activity-based protein profiling”²⁷) have attracted substantial attention.

Bio-Orthogonal Non-Canonical Amino Acid Tagging (BONCAT)

In 2006, Dieterich *et al* introduced the BONCAT (bio-orthogonal non-canonical amino acid tagging) method, a strategy for selective enrichment and identification of newly synthesized cellular proteins.¹⁵ The BONCAT method as originally introduced involved pulse-labeling of cells with the Met surrogate Aha. Cellular proteins made during the pulse were labeled with Aha and therefore susceptible to selective conjugation to alkyne-functionalized affinity probes via CuAAC (Fig. 2). Newly synthesized, affinity-tagged proteins can be enriched from the preexisting, unlabeled protein pool by affinity chromatography and identified by mass spectrometry. The BONCAT approach decreases sample complexity and permits direct analysis of the pool of proteins synthesized in response to biological cues.

As the BONCAT method was introduced, complementary techniques for visualization of newly synthesized proteins were also developed. One can visualize newly synthesized proteins in cells by appending fluorescent dyes, rather than affinity reagents, to metabolically labeled proteins. Cells labeled with Aha or Hpg can be selectively conjugated to fluorescent or fluorogenic dyes; imaging of intact cells reveals the spatial distribution of proteins synthesized during the ncAA pulse. We have demonstrated such methods in bacterial cells,²⁸ in mammalian cells,²⁹ and in live organisms. Fig. 3 compares mammalian cells pulsed with Aha under conditions that allow (top) or inhibit (bottom) the synthesis of new proteins. Robust labeling of new proteins is apparent in the absence of protein synthesis inhibitors, indicating that active protein synthesis is underway. The absence of signal in cells in which protein synthesis is inhibited confirms the selectivity of the method with respect to labeling of newly synthesized proteins. Multi-color tracking of two temporally distinct populations of proteins can be achieved by sequential tagging with Aha and Hpg,³⁰ and live-cell imaging of Aha-tagged proteins has been achieved by ligation to strained-alkyne dyes.³¹

NEW CHEMICAL AND GENETIC TOOLS FOR PROFILING PROTEIN SYNTHESIS

Increasing the sensitivity and selectivity of chemical reporter strategies constitutes a persistent challenge. Increased reaction yields with greater reproducibility³² as well as the design and synthesis of improved probes for visualization and enrichment³³ will extend the

limits of what can be detected. In this section we describe recent work directed toward the development of new chemical and genetic tools for the interrogation of cellular protein synthesis with reactive ncAAs.

An Alkene Reporter of Protein Translation

In 1998, van Hest et al reported the incorporation of homoallylglycine (Hag), an alkene-functional Met surrogate, into recombinant proteins.³⁴ Although it is charged to tRNA^{Met} more slowly than Aha or Hpg, Hag can replace Met quantitatively in proteins expressed in Met-auxotrophic strains of *E. coli*. At the time this work was reported, it was proposed that the Hag side chain might serve as a handle for bio-orthogonal protein modification. Recent developments have turned this proposal into reality. In 2009, Davis and coworkers described specific protein conjugation through radical addition of a glycosyl thiol to the Hag side chain.³⁵ Successful conjugation was demonstrated on a purified Hag-labeled protein, as well as on a virus-like scaffold displaying 180 surface Hag residues – in both cases, complete modification was observed. In a 2010 report, Song *et al* described the use of Hag to label newly synthesized proteins in mammalian cells.³⁶ Hag-labeled proteins were biotinylated, or conjugated to fluorescent dyes, via photo-induced tetrazole-alkene cycloaddition. In earlier work, the Lin group demonstrated the bio-orthogonality of the tetrazole-alkene cycloaddition by modifying a protein site-specifically labeled with *O*-allyl-tyrosine.³⁷

Engineering the aaRS

The natural biosynthetic machinery of microbial and mammalian cells permits the incorporation of a surprising number of ncAAs into cellular proteins. Nevertheless, there are substantial advantages to be gained by engineering the aaRS to expand the set of translationally active ncAAs.

In 2000, *in vivo* analysis in *E. coli* suggested that *trans*-crotylglycine (Tcg) could not support protein synthesis in a Met-auxotrophic strain depleted of Met. However, *in vitro* kinetic measurements revealed that Tcg was activated by the *E. coli* MetRS.³⁸ Kiick and coworkers proposed that the low rate of activation of Tcg limited the overall rate of protein synthesis, and that this limitation might be overcome through over-expression of the synthetase. In cells that expressed a plasmid-encoded wild-type MetRS (in addition to the genome-encoded copy), Tcg was successfully incorporated into protein.

Relaxation of synthetase specificity, either by attenuation of editing activity or by expansion of the amino acid binding site, can also enable incorporation of ncAAs. In nine of the twenty aaRSs, editing activity prevents misacylation of tRNA with non-cognate natural amino acids.⁹ Mutation in the *E. coli* leucyl-tRNA synthetase (LeuRS) editing domain (T252Y) attenuates the editing activity of the enzyme and permits incorporation of a variety of leucine (Leu) analogs,³⁹ including the ketone analog oxonorvaline (Onv).⁴⁰ The A294G mutation in the α -subunit of the *E. coli* phenylalanyl-tRNA synthetase (PheRS) relaxes substrate specificity by increasing the volume of the amino acid binding pocket.⁴¹ Over-expression of the mutant synthetase enables incorporation of a variety of reactive phenylalanine (Phe) analogs including *p*-bromophenylalanine (*p*-BrF), *p*-iodophenylalanine (*p*-IF), *p*-ethynylphenylalanine (*p*-EtF), and *p*-azidophenylalanine (*p*-AzF).⁴² However, over-expression of this mutant did not allow incorporation of *p*-acetylphenylalanine (*p*-AcF). Because of the versatility of ketone function in chemoselective ligations, we thought it worthwhile to seek a PheRS mutant that would enable its use in protein labeling. In a computational analysis, the crystal structure of the *Thermus thermophilus* PheRS complexed with Phe was used to construct a backbone-independent rotamer library. Calculations accurately predicted two cavity-forming mutations that would enable *p*-AcF activation.⁴³ A bacterial strain harboring the PheRS double mutant was prepared and shown to use *p*-AcF as

a Phe surrogate. *p*-AcF-labeled proteins were readily modified through hydrazide addition to the side-chain ketone.

The expression of mutant synthetases with altered substrate specificities expands the options available for protein labeling and proteomic analysis. For instance, in cells expressing the A294G mutant *E. coli* PheRS, *p*-AzF and *p*-EtF may be used as substitutes for – or complements to – Aha and Hpg in protein profiling. Tagging with analogs containing aryl halides, including *p*-BrF and *p*-IF is also possible.⁴² When first reported, these amino acids were proposed as handles for Pd-catalyzed cross-couplings; however, early efforts to adapt cross-coupling procedures to proteins in aqueous solvents were compromised by interference from cysteine thiols, required solvent degassing, and generally resulted in poor yields. In a promising recent development, Davis's adaptation of the Suzuki-Miyaura coupling employing a pyrimidine-Pd catalyst afforded specific and high-yield coupling of aryl bromide and aryl iodide groups on proteins.⁴⁴ Since Pd catalysts are poisoned by thiols, it is important that reduction and alkylation of cysteines (a standard step in sample preparation for proteomic analysis by mass spectrometry) be performed prior to addition of the Pd catalyst.

As described above, the toolkit for ncAA tagging has been expanded by the inclusion of two analogs containing ketones.⁴⁰ Chemoselective ligations to *p*-AcF or Onv residues in proteins can be achieved by treatment with hydrazide- or aminooxy-functionalized probes. While ketones and aldehydes are found on some cellular metabolites and cofactors, they are not normally found in proteins; complications in ligation reactions can be avoided by first separating protein from other cellular constituents. However, Bertozzi has suggested that the intracellular presence of ketone-containing metabolites may restrict dye-labeling and imaging applications of ketone- and aldehyde- labeled biomolecules to the cell surface where these functions are usually absent.⁴⁵

Combinatorial Approaches to Mutant aaRS

Efficient screening and selection methods have been developed to enable rapid identification of aaRS mutants of altered substrate specificity.^{6, 46, 47} Much of our recent effort in this area has been directed toward isolation of MetRS mutants that activate azidonorleucine (Anl), a long-chain variant of Aha. Using structure-guided mutagenesis, we constructed a library of MetRS mutants by randomization at three positions (L13, Y260 and H301) adjacent to the amino acid binding site. Clones expressing functional mutants were selected on the basis on their ability to incorporate Anl into the outer membrane protein OmpC (Fig. 4a).^{48, 49} Azides displayed on the cell surface were detected by ligation to a biotin-cyclooctyne reagent and treatment with a fluorescent avidin conjugate. Bright clones were isolated by fluorescence-activated cell sorting (FACS). The FACS screen uses the extent of fluorescence labeling as a reporter for Anl incorporation, and in turn, for MetRS activity toward Anl; the highest levels of OmpC expression and fluorescence labeling are expected in cells carrying MetRS mutants most active toward Anl. Through this approach, more than 40 different MetRS mutants were identified, each allowing incorporation of Anl into proteins with varying efficiency.^{47, 50} A combination of mutational and x-ray crystal structure analyses showed that the successful mutations eliminated hydrophilic contacts with the Met side chain, thereby reducing competition with Met and polar amino acids while creating a hydrophobic pocket for Anl (Fig. 4b, 4c).⁵¹ The L13N/Y260L/H301L mutant (NLL-MetRS) proved to be especially interesting, in that its specificity constant (k_{cat}/K_M) for activation of Anl was found to be slightly larger than that for Met.

Cell-selective Metabolic Labeling of Proteins

Access to the NLL-MetRS mutant allowed us to develop a method for cell-selective proteomic analysis.⁵² Cells that express the NLL-MetRS can utilize Anl in protein synthesis; cells that do not express the mutant synthetase are inert to Anl. In a cellular mixture, only proteins from mutant cells are labeled. Through this approach, proteins synthesized in targeted cells can be selectively isolated from complex mixtures for identification by mass spectrometry or conjugated to fluorescent dyes for in situ visualization. In a first demonstration of the method, we confined protein labeling with Anl to bacterial cells co-cultured with mammalian macrophages (Fig. 5). Careful analysis of bacterial proteins in such mixtures requires enrichment because of the disparity in the sizes and protein contents of bacterial and mammalian cells; bacterial proteins typically account for less than 1% of the total protein content in an infection mixture.⁵³ Anl-tagged proteins were selectively ligated to biotin and separated from mammalian proteins in an infection lysate (Fig. 3). The Hang laboratory has used the NLL-MetRS to activate the alkyne analog, 2-aminooctynoic acid (Aoa) for cell-selective labeling.⁵⁴ The authors used Aoa to label bacterial proteins expressed in macrophage-internalized *Salmonella typhimurium*. While the NLL-MetRS was isolated from a screen for mutants active toward Anl, the Hang experiments show that it also activates Aoa. NLL-MetRS mediated labeling with Aoa required removal of Met from the culture medium, whereas Anl-labeling did not. Hang and coworkers found lower background in reactions between azide-functionalized probes and Aoa-tagged proteins than in parallel experiments with alkyne detection reagents and Anl-tagged proteins.

Some Considerations

It is important to keep in mind that profiling technologies are intended to aid and accelerate *discovery*. Proteins identified in profiling experiments of the kind discussed here may be involved in the biological process of interest, but may be bystanders or products of secondary effects. The possibility that artifacts can arise in labeling experiments is well known, and should be a source of caution in interpreting results. In the methods described here, ncAAs are incorporated into many proteins, with unknown effects on cellular behavior. Artifacts are likely to be minimized by short labeling times and modest labeling rates, and since the methods used to track labeled molecules are sensitive (fluorescence microscopy and mass spectrometry), labeling rates of a few percent are sufficient. The labeling rate is easily controlled by adjustment of the relative concentrations of the amino acid to be replaced and the ncAA reporter.

RECENT APPLICATIONS

ncAA-tagging strategies have been employed in recent years to interrogate several complex cellular processes. Examples include kinetic analysis of protein-modification and turnover rates, and imaging of newly synthesized proteins in neurons.

Deal and coworkers have reported an ncAA-tagging method (designated CATCH-IT, for covalent attachment of tags to capture histones and identify turnover) to examine the dynamics of genome-wide nucleosome disassembly and reassembly, which are important in epigenome maintenance.⁵⁵ In the CATCH-IT approach, newly translated histones are tagged with Aha and enter the nucleus where they are incorporated into chromatin. After tagging, total chromatin is isolated, and tagged nucleosomes are ligated to biotin probes. Newly synthesized nucleosomes are isolated by binding to streptavidin beads, and the associated DNA is identified on microarrays to determine the genome positions at which old histones have exchanged with new.

Zhang and colleagues have combined tandem labeling (with Aha and an alkyne analog of palmitic acid) and fluorescence imaging to examine S-palmitoylation dynamics in Jurkat

cells.⁵⁶ The rate of turnover of H-RasG12V (determined from the Aha signal intensity in a pulse-chase experiment) was correlated with fatty acid cycling (from the palmitate analog). The results were consistent with dynamic S-acylation and minimal protein turnover within the 5-hour time interval examined.

Local protein synthesis in neuronal dendrites is thought to contribute to learning and memory, and provided the initial motivation for development of the BONCAT method. This process has been examined in a recent report by Dieterich and colleagues in which Aha and Hpg were used to visualize protein synthesis in dissociated rat hippocampal neurons and in organotypic slices.⁵⁷ Micro-perfusion of the ncAA permitted direct detection of local protein synthesis in neuronal dendrites. The diffusion dynamics of newly synthesized proteins were examined by conjugation of ncAA-tagged proteins to quantum dots.

In recent work by Flanagan and coworkers, Aha was used to visualize local protein synthesis in specific sub-regions of the neuronal cytoplasm in response to external cues.⁵⁸ DCC (deleted in colorectal cancer) is a transmembrane receptor that regulates protein translation in axons and dendrites in response to the extracellular ligand netrin. Because DCC colocalizes with components of the translational machinery in neuronal dendrites and axons, it was thought that it might regulate local protein synthesis in the neuron. Since it is unknown which mRNAs are translated in response to netrin binding, individual proteins could not be examined for local up-regulation. To probe the relation between netrin binding and local protein synthesis, Flanagan and coworkers used Aha to tag proteins made in netrin-stimulated neurons. Aha-tagged proteins were found throughout the neuron, but exhibited discrete colocalization with DCC along axons, within dendrites and at the tips of filopodia. Aha-tagging was used to measure variations in protein synthesis activity within DCC microdomains by quantifying fluorescence from dye-labeled neurons. Netrin-stimulated cells showed marked increases in protein synthesis in DCC microdomains, further supporting a role for netrin binding in the positive regulation of local translation.

FUTURE DIRECTIONS

While there are many promising applications of ncAAs in protein engineering and molecular medicine^{7, 59, 60}, this Account has focused on the incorporation of reactive ncAAs and their use as analytical tools in chemical biology. We continue to develop ncAA-tagging strategies by creating new ncAAs, engineering new aaRS mutants, and preparing new probes. In collaboration with colleagues, we are pursuing studies of host-pathogen interactions and cell signaling, as well as methods for interrogating protein synthesis in live animals.

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BIOGRAPHICAL INFORMATION

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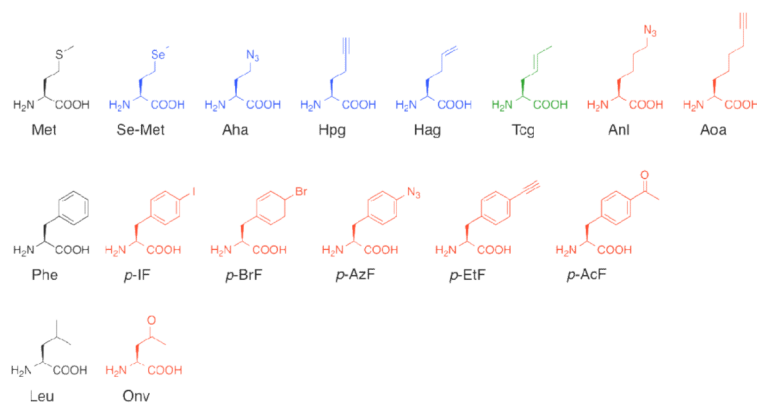
returned to UMass in 1984, and in 1998 moved to Caltech, where he chaired the Division of Chemistry and Chemical Engineering from 1999 until 2009.

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**Figure 1.**

Structures of the amino acids discussed in this Account. ncAAs shown in blue are substrates for the natural translational machinery, the analog shown in green requires over-expression of wild-type MetRS, and those shown in red require expression of mutant aaRSs.

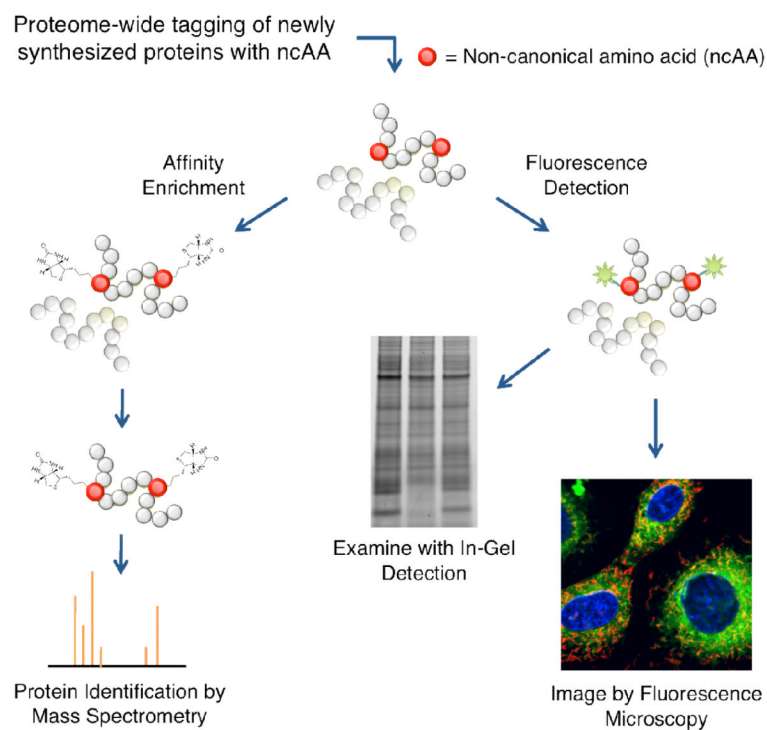


Figure 2. ncAA-tagged proteins can be ligated to affinity probes for enrichment¹⁵ and identification, or to dyes for visualization by in-gel fluorescence scanning or fluorescence microscopy.²⁹

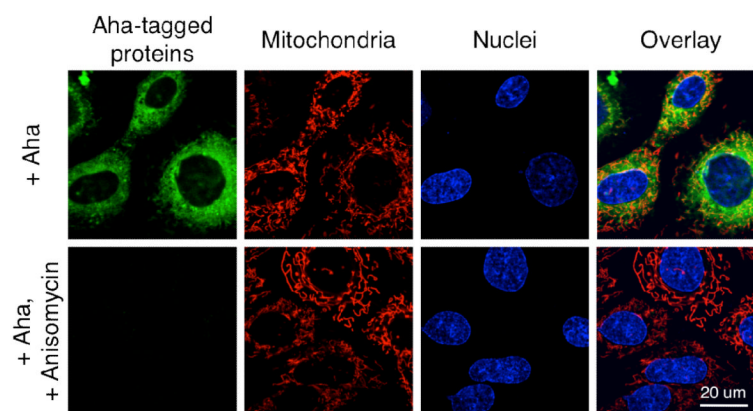


Figure 3.

Imaging of mammalian cells pulsed with Aha in the absence (top) or presence (bottom) of the protein synthesis inhibitor anisomycin. The left-most panel in each row shows the image formed by dye-labeling of newly synthesized proteins (green). Additional panels show mitochondria (red), nuclei (blue), and panel overlays.

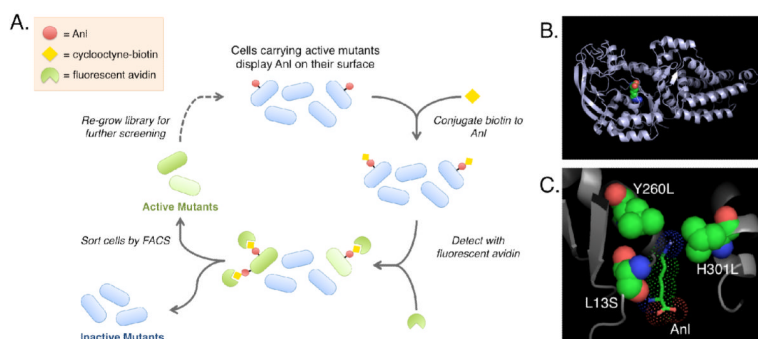
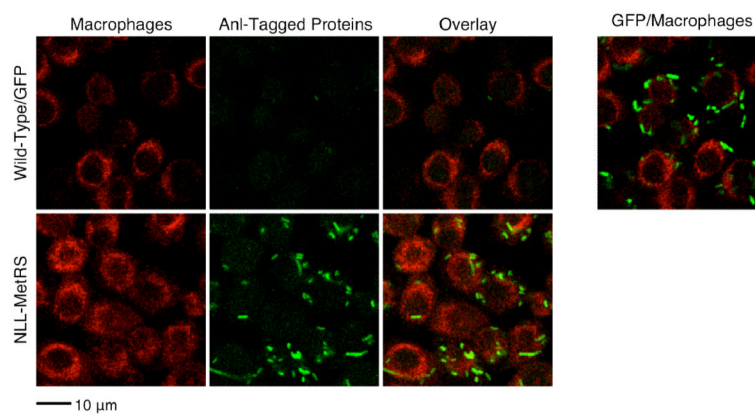


Figure 4.

(a.) Library screen for MetRS mutants that allow Anl incorporation into cellular proteins.^{47, 50} (b.) X-ray crystal structure of a MetRS mutant (L13S/Y260L/H301L) bound to Anl (spheres).⁵¹ (c.) Mutations (spheres) that accommodate Anl (sticks and dots) within the MetRS binding pocket.⁵¹

**Figure 5.**

Cell-selective labeling in mixtures of bacterial and mammalian cells.⁵² Macrophages (red) infected by *E. coli* cells that express GFP (top) or the NLL-MetRS (bottom) labeled with Anl. Proteins expressed in bacterial cells that express the NLLMetRS are labeled with Anl. The control bacterial strain was bound and internalized by macrophages (as confirmed by detection of GFP) but exhibited no Anl incorporation. In neither case were macrophage proteins labeled.